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# S3226, a novel NHE3 inhibitor, attenuates ischemia-induced acute renal failure in rats

**MAX HROPOT, HANS-PAUL JURETSCHKE, KARL HEINZ LANGER, and JAN-ROBERT SCHWARK**

*Aventis Pharma Deutschland GmbH, Frankfurt am Main, Germany*

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*Background.* Acute renal failure (ARF) remains a major problem in clinical nephrology characterized by sudden loss of the kidney function due to ischemia, trauma, and/or nephrotoxic drugs. The current therapy of ARF is symptomatic with mortality rates exceeding 50%. The aim of this study was to investigate the effects of an intravenous infusion of S3226 (3-[2-(3-guanidino-2-methyl-3-oxopropenyl)-5-methyl-phenyl]-N-isopropylidene-2-methyl-acrylamide dihydrochloride), a selective $Na^+/H^+$ exchange subtype 3 (NHE3) blocker, in ischemia-induced ARF in rats. In a second series of experiments cytosolic pH (pHi) changes in the kidney during ARF were continuously measured by means of nuclear magnetic resonance spectroscopy (MRS).

*Methods.* ARF was induced by bilateral occlusion of renal arteries for 40 minutes in three groups of anaesthetized Wistar rats. Control rats ($N = 12$) were infused with saline (6.25 mL/kg over 30 min) before occlusion and the compound groups (each $N = 12$) were infused with S3226 at a dose of 20 mg/kg over 30 minutes either before initiation of ischemia or immediately after release of clamps. Plasma creatinine ($P_{Cr}$), creatinine clearance ($C_{Cr}$), urine volume, sodium, and potassium excretion were determined up to seven days after release of clamps. In the second series of experiments in anaesthetized rats the left kidney was exposed by flank incision and fixed in a non-magnetic device. An inflatable cuff was positioned around the pedicle to induce ischemia without removing animals from the magnet. A double-tuned $^1H$-$^{31}P$ home-built surface coil was placed above the exposed kidney for the detection of pHi.

*Results.* At day 1 after ischemia $C_{Cr}$ in the control group was significantly lower as compared to S3226-treated animals (control 0.30 ± 0.05 vs. before 0.90 ± 0.26 and reperfusion 0.83 ± 0.15 mL/min/kg, respectively). $P_{Cr}$ increased from 18 ± 0.1 μmol/L before occlusion to 245 ± 7 μmol/L in the control. The increase in $P_{Cr}$ was significantly lower in the S3226 treated groups on days 1, 2, and 3 post-infusion. Fractional sodium excretion decreased significantly from 8.17% in the control to 1.42% and 1.88% in the treated groups. Renal pHi was significantly decreased by 0.15 units versus control during reperfusion. Histological examination of the kidneys on day 7 revealed pronounced reduction of tubular necrosis, dilatation, protein casts and cellular infiltration.

*Conclusions.* These results demonstrate that an intravenous administration of S3226 acutely improves GFR and kidney function and structure in both treated groups. In addition, in a separate set of studies S3226 significantly decreased post-occlusion renal pHi values. Thus, the inhibition of NHE3 with S3226 may be beneficial in treatment of ischemic ARF.

**Key words:** sodium-hydrogen-3 blocker, creatinine clearance, $^{31}P$-magnetic resonance spectroscopy, cytosolic pH, phosphate metabolism, urinary excretion.

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Acute renal failure (ARF) is a serious disease with a patient mortality exceeding 50 percent. For decades therapeutic maneuvers have been symptomatic including mannitol, loop diuretics alone or in combination with dopamine, dialysis and other supportive treatments [1, 2]. Apart from small-sized clinical studies examining atrial natriuretic peptides (ANP) in ARF that have demonstrated improved creatinine clearance and reduced need for dialysis, there is no treatment modality to date that has provides a significant improvement of ARF [3].

Recently, several animal studies have indicated that different classes of chemical compounds ameliorate the outcome of ischemic and nephrotoxic ARF. It has been shown that ANP infusion has beneficial effects on norepinephrine- and ischemia-induced ARF [4, 5]. Furthermore, it has been demonstrated that xanthine derivatives such as KW-3902 and CVT-124, which are adenosine $A_1$-receptor antagonists, significantly ameliorate cisplatin-, gentamicin- and ischemia-induced ARF in rats. As possible mechanisms of action, the natriuretic and renal hemodynamic effects of these compounds have been discussed as beneficial principles in ARF [6, 7]. Furthermore, it has been demonstrated that an adenosine analog highly selective for $A_{2A}$-receptors, the agonist DWH-146e, significantly ameliorated ischemia-induced ARF [8]. Moreover, it has been suggested that endothelin may be involved in the pathophysiology of ischemic ARF. Elevated plasma levels of endothelin were reported in experimental and human acute renal failure (abstract; Tomita et al, *N Engl J Med* 321:1127, 1989]. Since it has been shown that $Na^+/H^+$ exchanger subtype 3 (NHE3) is involved in renal ischemia-reperfusion injury, it could be postulated that a

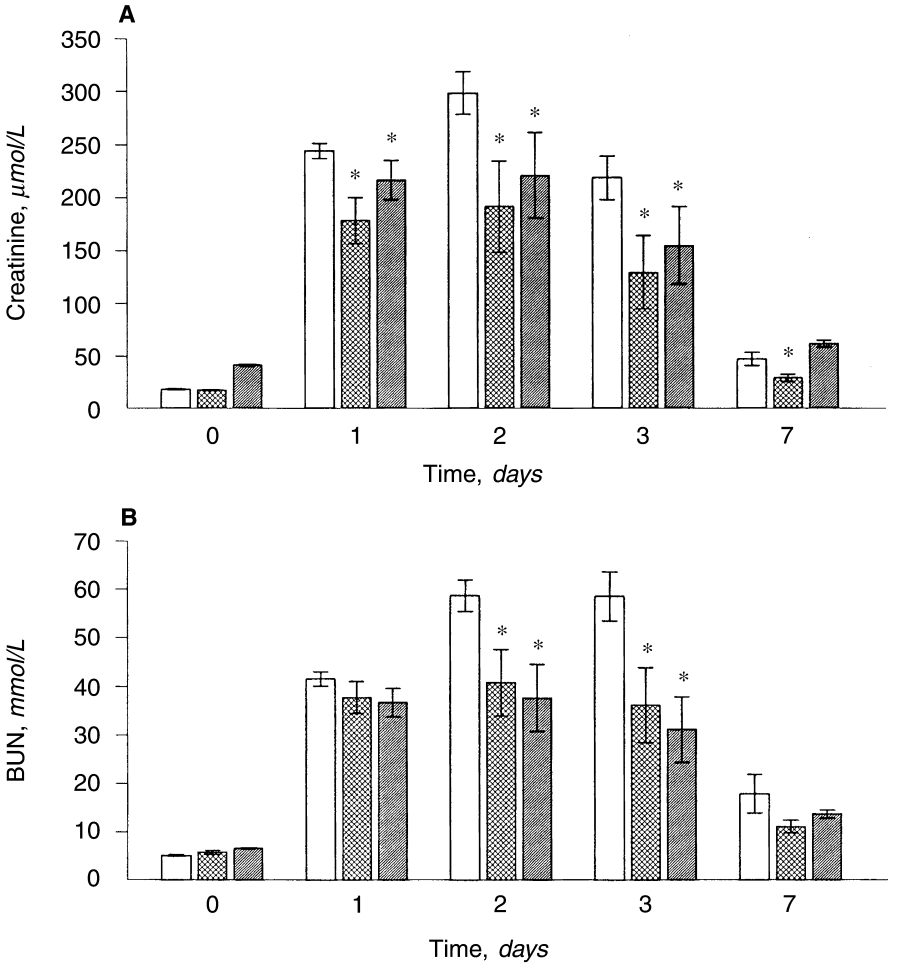


**Fig. 1. Effects of intravenous infusion of S3226 in acute renal failure (ARF) on plasma creatinine (*A*), blood urea nitrogen (BUN; *B*), sodium (*C*) and potassium (*D*).** Renal arteries were occluded for 40 minutes. S3226 was infused for 30 minutes before ischemia (▨; $N = 12$) or after release of clamps (■; $N = 12$) at a dose of 20 mg/kg. Control rats received saline in a volume of 6.25 mL/kg (□; $N = 12$). Values are means ± SEM, $^*P < 0.05$ vs. control group.

direct inhibition of this exchanger with a selective NHE3 inhibitor could improve ARF [10].

Thus, the purpose of the present study was to examine the effects of the NHE3 inhibitor S3226 in ischemia-induced ARF in rats [11]. In addition renal pHi changes in normoxic and ischemic kidney being treated with S3226 were examined by means of nuclear magnetic resonance spectroscopy (MRS). We found that NHE3 inhibition significantly improved the outcome of ARF in rats and that this inhibition also reduced the renal pHi.

## METHODS

### Animals

Experiments were performed in male Wistar rats with a mean body weight of 285 ± 7.5 g. Animals were supplied by Aventis Tierhaltung and were kept in an air-conditioned animal housing in a 12-hour light/dark cycle at 22°C and fed a standard diet (Altromin®) and had access to tap water ad libitum. All experiments were performed in accordance with the German Animal Protection Law.

### Renal ischemia-reperfusion model

The animals were randomly allocated to control and two compound groups. During experiments the body temperature was kept constant (37°C) by placing animals on a heating pad. Rats were anaesthetized by Ketavet® and Rompun® at doses of 100 and 5 mg/kg i.m., respectively. Kidneys were exposed through a flank incision and renal arteries and veins were clamped with artery clamps for 40 minutes (Aesculap® yasargil-clip, closing force 0.98 to 1.28 N). Control rats received 0.9% NaCl solution intravenously in a volume of 6.25 mL/kg body weight for 30 minutes before occlusion. S3226 was dissolved in saline (20 mg/kg body weight) and infused in a volume of 6.25 mL/kg body weight before occlusion for 30 minutes. The second compound group received S3226 (20 mg/kg for 30 min) immediately after release of clamps. After termination of acute experiments rats were monitored for seven days. On days 0, 1, 2, 3 and 7 blood samples were collected retro-bulbary (in short inhalation anesthesia) for determination of creatinine, urea, sodium, potassium, and albumin. In addition, on days 1 and 7 diuresis experiments were performed to

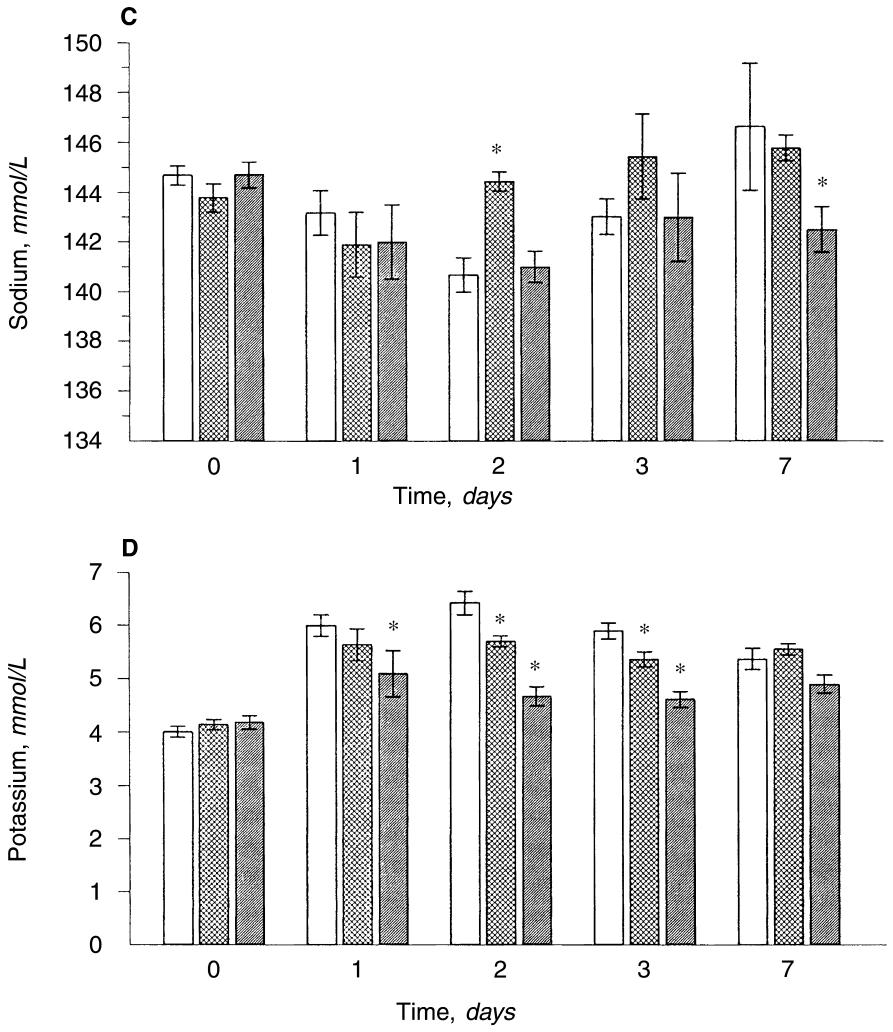


**Fig. 1. (Continued)**

assess the excretory kidney function. Sodium and potassium in urine and plasma samples were measured by flame photometry (Photometer Eppendorf, Hamburg, Germany), and creatinine and urea were determined using the Crea plus® and the Urica-quant® method, respectively. The basis for selecting 20 mg/kg of S3226 was its in vitro $IC_{50}$ of 0.2 μmol/L in fibroblasts transfected with rat NHE3. The concentration measured in the intravascular compartment after treatment was 2 μmol/L and $t_{1/2}$ = 30 minutes.

### Measurement of renal pHi by means of MRS

*Second series of experiments.* One week after unilateral nephrectomy rats were anaesthetized with Inactin® (150 mg/kg, IP) and the remaining kidney was exposed by flank incision and immobilized in a home-built device. Body temperature was maintained constant (37°C) with a heating water pad. An inflatable cuff was positioned around the renal pedicle to induce ARF without removing the rat from the magnet. A double-tuned $^{1}H$-$^{31}P$ home-built surface coil was placed above the exposed kidney for signal detection. A thin copper foil was placed between the kidney and the animal's body to minimize signal interferences originating in adjacent muscles and skin tissue. $^{31}P$ MRS measurements were performed at 4.7 Tesla on a Biospec® spectrometer (Bruker Medical, Ettlingen, Germany). $^{31}P$ NMR spectra exhibiting the signals of ATP, phosphodiesters, inorganic and sugar phosphates were collected at 81.1 MHz with 600 scans, spectral width of 8000 Hz, data size of 512 points, pulse width of 50°, acquisition time of 0.028 s, and a recycle time of 0.4 s. With these parameters good quality spectra were obtained within four minutes. As in kidney $^{31}P$ NMR spectra no phosphocreatine peak is visible, the changes in chemical shift of the inorganic phosphate (Pi) resonance, were related to the signal of an external standard (diphenylphosphonic acid contained in an adjacent capillary) to derive intracellular pHi. The pHi was calculated according to the formula: $pH = 6.75 + \log(d - 3.06)/(5.75 - d)$, where d is the difference in chemical shift between the resonances of Pi and phosphocreatine [12]. The compound S3226 was infused at the dose of

**Table 1.** Effects of drug treatment on urine output, urinary excretion of sodium potassium and albumin, glomerular filtration rate and fractional excretion of sodium and potassium

| | Vehicle group | | S3226 before occlusion *20 mg/kg per 30 min* | | S3226 in reperfusion *20 mg/kg per 30 min* | |
|---|---|---|---|---|---|---|
| | 1 day a. t. $N = 12$ | 7 days a. t. $N = 9$ | 1 day a. t. $N = 12$ | 7 days a. t. $N = 12$ | 1 day a. t. $N = 12$ | 7 days a. t. $N = 12$ |
| Urine output *mL/kg* | 16.71 ± 1.75 | 17.63 ± 2.16 | 20.49 ± 2.61 | 15.79 ± 1.40 | 17.57 ± 3.79 | 18.45 ± 3.61 |
| $C_{Cr}$ *mL/min/kg* | 0.30 ± 0.05 | 3.22 ± 0.37 | 0.90 ± 0.26[a] | 4.03 ± 0.54 | 0.83 ± 0.15[a] | 4.57 ± 1.07 |
| $U_{Na}V$ *mmol/kg* | 0.98 ± 0.17 | 0.28 ± 0.08 | 0.31 ± 0.08[a] | 0.57 ± 0.13[a] | 0.31 ± 0.06[a] | 0.45 ± 0.11[a] |
| $U_KV$ *mmol/kg* | 0.97 ± 0.08 | 1.60 ± 0.18 | 1.17 ± 0.14 | 2.10 ± 0.17 | 0.85 ± 0.16 | 3.11 ± 0.95 |
| $FE_{Na}$ % | 8.17 ± 1.30 | 0.21 ± 0.06 | 1.42 ± 0.45[a] | 0.38 ± 0.11 | 1.88 ± 0.41[a] | 0.35 ± 0.09[a] |
| $FE_K$ % | 198.67 ± 13.44 | 32.22 ± 3.11 | 125.55 ± 23.41[a] | 34.17 ± 3.61 | 141.31 ± 23.71[a] | 37.21 ± 5.37 |
| $U_{Albumin}V$ *mg/24 h* | — | 0.42 ± 0.03 | — | 0.29 ± 0.04 | — | 0.33 ± 0.06 |

Values are means ± SEM. Abbreviations are: $U_{Na}V$ and $U_KV$, sodium and potassium excretion rates; $FE_{Na}$ and $FE_K$, fractional excretion of sodium and potassium; a. t., after treatment. Urine samples were collected by placing the rats in diuresis cages for 5 hours. S3226 was infused for 30 min before occlusion and after release of clamps, respectively.

[a] $P < 0.05$ to corresponding vehicle group value

20 mg/kg over 30 minutes before occlusion and a second time starting five minutes before release of clamps, respectively. The rationale for two treatments was a pharmacokinetic study which revealed that only 10% of the parent compound was renally excreted while the major part of S3226 was metabolized in the liver. To achieve a sufficient concentration of the compound at the renal site of action, in this series of experiments S3226 was infused before and after induction of renal ischemia.

### Histological evaluation

For light microscopy all kidneys were removed in anesthesia on day 7 of reperfusion and fixed in 10% neutral buffered formalin and embedded in paraffin. Three to 4 μm sections were stained with hematoxylin and eosin (H&E) or with periodic acid Schiff (PAS) stain.

### Statistical analysis

Values are expressed as arithmetical mean ± SEM. A one-way ANOVA was calculated with SYSTAT for Windows (SYSTAT, Inc., Evanston, IL, USA) followed by multiple pairwise comparisons according to Tukey. Null hypotheses were rejected at $P < 0.05$.

## RESULTS

After 40 minutes of bilateral renal artery occlusion and 24 hours of reperfusion, $P_{Cr}$ (plasma creatinine) and BUN significantly increased as compared to initial values ($P < 0.05$). On day 2 there was a further increase of $P_{Cr}$ and BUN which then gradually decreased on days 3 and 7 (Fig. 1 A, B). In parallel, creatinine clearance ($C_{Cr}$) dramatically decreased to 0.30 mL/min/kg body wt 24 hours after the release of occlusion as compared to our routine laboratory values of 6 to 8 mL/min/kg in healthy rats. Infusion of S3226 in a dose of 20 mg/kg for 30 minutes prior to occlusion and after release of clamps significantly reduced $P_{Cr}$ and BUN and significantly augmented $C_{Cr}$ to 0.90 and 0.83 mL/min/kg on day 1 as compared to controls (Table 1 and Fig. 1). Urine excretion was not different between days 1 and 7.

The impairment of renal function also was reflected by the changes in sodium and potassium concentration in plasma and urine. Plasma sodium was markedly reduced in control and both compound groups on day 1 as compared to initial values. However, treatment with the NHE3 blocker S3226 normalized the plasma sodium concentration already at day 2. Plasma potassium concentration was significantly increased in all groups on day 1 in comparison with the initial values and was significantly lower in the treated groups on days 2 and 3 as compared to the control group (Fig. 1 C, D). Urinary sodium excretion was significantly reduced in the treated groups as compared to the control (0.31 ± 0.08, 0.31 ± 0.06 vs. 0.98 ± 0.17 mmol/kg body weight) on day 1 (Table 1). In parallel fractional sodium excretion ($FE_{Na}$) was 8.17% in the ischemic control versus 1.42% and 1.88% in the treated groups on day 1 and decreased further on day 7. Urinary potassium excretion was not different on days 1 and 7, whereas $FE_K$ (fractional potassium excretion) was higher in the control group at day 1 as compared to the treated groups. $FE_K$ was not different between groups at day 7 (Table 1). Urinary albumin excretion as a marker of glomerular lesions was measured on day 7 and revealed a slight reduction in the treated groups as compared with the control group. Furthermore, in the control group 3 rats died on day 4, whereas all animals survived in the treated groups, however, the difference was not statistically significant.

To assess renal pHi in ARF cytoplasmatic signals of Pi and other phosphate metabolites were measured by NMR spectroscopy in vivo. Intravenous infusion of S3226 according to protocol did not change the renal pHi in sham operated rats as compared to vehicle control (Fig. 2A). On the other hand, a 40 minute occlusion of the renal artery caused a profound drop in pHi which

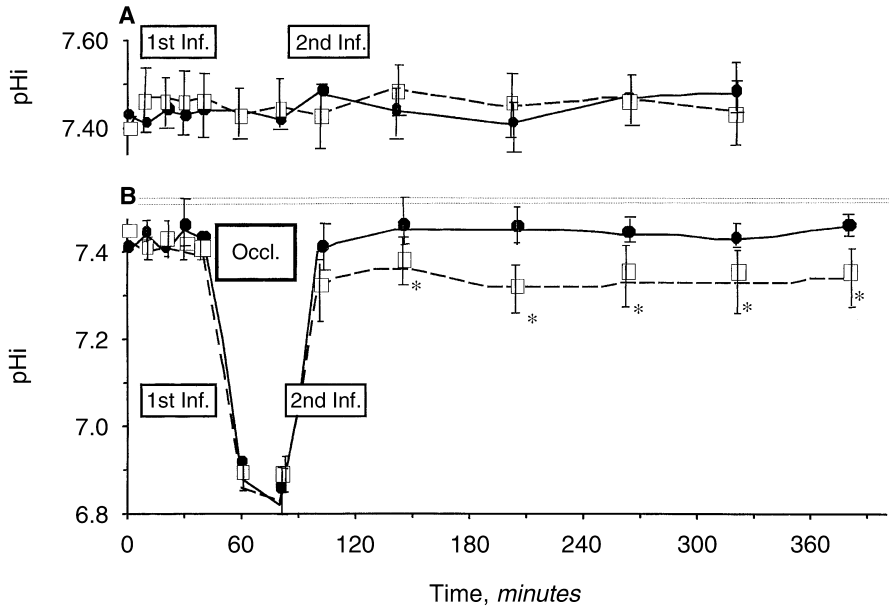


**Fig. 2. Effect of S3226 on renal intracellular pH of fasted uninephrectomized rats as monitored by in vivo $^{31}$P NMR spectroscopy.** The drug was infused twice to maintain suitable blood levels as indicated in the labeled boxes at a dose of 20 mg/kg over 30 minutes. (*A*) S3226 did not affect pHi during normoxia ($N = 4$ each group). (*B*) S3226 was infused prior to and 5 minutes before ending of ischemia. In the S3226 group ($N = 8$ for each group) pHi was significantly reduced by 0.15 units ($P < 0.05$) during the 5-hour reperfusion period. Values are means ± SEM. Symbols are: (●) control, (□) drug. *$P < 0.05$ vs. control group.

quickly recovered to the initial values as shown in Figure 2B. However, after infusion of S3226 twice, before occlusion and five minutes before release of clamps each for 30 minutes, the pHi remained significantly below the pre-ischemic and vehicle values during the observation time of five hours. At the same time there was a dramatic reduction of renal adenosine 5′-triphosphate (ATP) in the ischemic period which recovered post-ischemically though without reaching its pre-ischemic value (not shown). There was no significant difference in ATP concentration in the control versus drug group after induction of ischemia.

Histological examination of the kidneys on day 7 revealed pronounced reduction of tubular necrosis, dilatation and protein casts, and cellular infiltration of the broadened peritubular interstitium in the treated groups (Fig. 3B) as compared to the vehicle group (Fig. 3A).

## DISCUSSION

Intravenous infusion of the NHE3 inhibitor S3226 prior to renal artery occlusion and in reperfusion dramatically improved the outcome of ARF in rats. There was a significant reduction of plasma creatinine and BUN concentration and a modest but significant increase of $C_{Cr}$ 24 hours after administration of S3226. In the past, several experimental studies have demonstrated that it was possible to improve the course of ischemic ARF by using agents such as atrial natriuretic factor (ANF), adenosine $A_1$ antagonist and $A_2$ agonists, endothelin $ET_A$ antagonists, as well as the nitric oxide (NO) donor FK409 [4–6, 8, 14–16]. Atrial natriuretic peptide (ANP) has been shown to reverse experimental models of norepinephrine- and ischemia-induced ARF [4, 5]. Glomerular filtration rate (GFR), renal blood flow as well as urine and sodium excretion were significantly improved in comparison with vehicle groups. ANP has been shown to increase GFR in pathophysiological states by altering pre- and post-glomerular vascular resistance resulting in enhancement of the glomerular capillary hydraulic pressure. Furthermore, in a small sized study in ARF patients it was shown that short-term parenteral infusion of ANP caused an increase in $C_{Cr}$ and that the need for dialysis was significantly reduced [3].

S3226 is a potent NHE3 inhibitor in vitro, as shown in transfected fibroblasts [11]. In a micropuncture study, microperfusion of proximal convoluted tubules with S3226 showed a dose-dependent 30% inhibition of fluid and sodium absorption [16]. On the other hand, HOE 642, a selective NHE1 inhibitor, did not alter fluid and sodium absorption in proximal convoluted tubules. As the luminal $Na^+/H^+$ exchanger NHE3 plays a prominent role in proximal fluid and sodium reabsorption, its contribution was assessed by micropuncture in NHE3 knockout mice. It was demonstrated that proximal fluid reabsorption was blunted in this species indicating that NHE3 is the major NHE isoform mediating sodium and fluid transport in the proximal tubule [17, 18]. In our experiments ischemic ARF caused $FE_{Na}$ of 8.17%, which was significantly reduced to 1.42 and 1.88% after administration of S3226 in both treated groups. This indicates that administration of S3226 improves tubular function and recovery already at day 1. In parallel, the diminished plasma sodium concentration was restored to normal. At the same time plasma potassium and $FE_K$ were significantly decreased as compared to vehicle control indi-

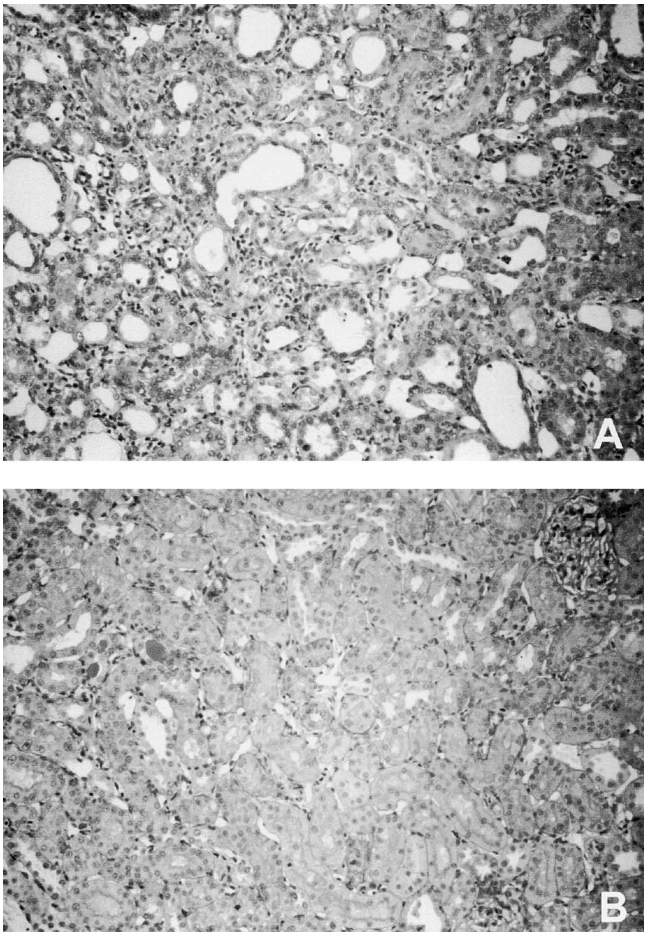


**Fig. 3. Overview of the kidney cortex from a control (*A*) and a treated (*B*) rat.** In panel A, tubular single or group necrosis (often extruded into the lumen), tubular dilation and diffusely distributed round cell infiltration in a broadened interstitium are visible, but these lesions are not observed in panel B. PAS staining, magnification ×160.

cating that NHE3 inhibition may contribute to restoration of the proximal tubular transport process.

Recently, a great body of evidence has accumulated implicating endothelin peptides (ET) in pathophysiology of ARF. The vasoconstrictor properties of ET not only affect vascular tone, but also influence tubular sodium and water transport [19]. It has been demonstrated that the selective $ET_A$ receptor blockers LU-135252 and BQ-123 possess beneficial effects in ischemic ARF due to increased renal perfusion and attenuation of intracellular calcium accumulation in the early phase of ischemic ARF [13, 14].

Cytosolic $[Ca^{2+}]$, $[H^+]$, and $[Na^+]$ are closely linked to NHE3 activity. The increased sodium load would lead under conditions of reduced ATP supply during ischemia to a reversed action of $Na^+/Ca^{2+}$ exchanger resulting in an increased intracellular calcium with its detrimental effects. Therefore, in a second series of experiments we evaluated changes in renal pHi with the MRS technique during infusion of S3226. During an observation time of five hours (reperfusion period) the renal pHi was significantly lower in the treated group as compared to the vehicle group. This delayed restoration of pHi as a result of NHE3 inhibition prevents the cell from sodium load.

In the post-acute phase it has been shown by Wang et al. that 30 minutes of renal artery occlusion caused approximately 70% decrease in NHE3 mRNA and NHE3 activity, resulting in overexpression of colonic type $H^+/K^+$-ATPase in the cortex to compensate the reabsorption of increased bicarbonate load resulting from suppression of NHE3 [10]. However, the activity of remaining NHE3 transporters may be increased by the cytosolic acidification that occurs in the occlusion phase and in addition, reperfusion could activate NHE3 again. Thus, inhibiting NHE3 may improve the outcome of ARF. On the other hand, it has been demonstrated that metabolic acidosis caused increased expression of renal brush border NHE3 protein resulting in normalization of pHi during metabolic acidosis [20–22]. Therefore, a sustained cellular acidosis could accelerate recovery of the cell function.

In the present study, the S3226 treated groups revealed pronounced reduction of tubular necrosis and cellular infiltration as well as survival of all rats in this groups as compared to the vehicle group (3 rats out of 12 died before termination of the experiment), however the difference was not statistically significant. A distinct preservation of the normal tubular architecture was also shown by others employing the platelet-activating factor (PAF) antagonist Ro-244736, the spontaneous NO donor FK-406, and the selective $A_{2A}$-adenosine receptor agonist DWH-146e, [8, 15]. PAF is vasoconstrictive and promotes platelet aggregation and its role in leukocyte-endothelial adhesion and leukocyte extravasation following reperfusion was postulated. Moreover, it has been demonstrated that oral administration of losartan, an angiotensin II receptor subtype 1 antagonist was able to improve the outcome of ischemic ARF by reducing plasma creatinine, and proteinuria and increasing the survival rate [abstract; Heller et al, *Kidney Int* 55(Suppl):S113, 1996]. This effect is likely linked to NHE3 activity since previous observations demonstrated that intrarenal angiotensin II (Ang II) dose-dependently regulates the luminal NHE3 possibly by protein kinase C stimulation and a reduction of cAMP production [23].

On the other hand, adenosine receptors are known modulators of renal haemodynamics and potent inhibitors of inflammation. It has been reported that adenosine $A_1$-receptor antagonists reduce renal injury in models of ARF by decreasing adenosine-mediated vasoconstriction of afferent arterioles [6, 7]. In addition, the pronounced diuretic effect of these compounds was attributed to their interaction with the NHE3 via cAMP accumulation. Another interesting compound, which

was shown to improve markedly the outcome of ischemic ARF, is the adenosine $A_{2A}$-receptor agonist DWH-146e. This compound was infused via minipumps following release of clamp causing a significant reduction of plasma creatinine, inhibition of inflammation and increase in urine osmolality [8].

In summary, the present results demonstrate that S3226, a selective NHE3 inhibitor, exerts protective effects on kidney function and structure during ischemic ARF. These effects achieved in two sets of studies include amelioration of GFR, $FE_{Na}$ and $FE_K$, as well as a decrease in renal pHi. The demonstrated pharmacological effects are obviously the cause for reduced cellular necrosis in proximal tubules. The observed interstitial reparative processes in treated rats are in contrast to those in the untreated animals in which the full recovery of the kidney function is uncertain because of the more or less diffuse degeneration status at day 7. No mortality in the treated rats but decreased survival rate in the untreated rats corresponds to the observed histological findings. Thus, the inhibition of NHE3 with S3226 may be beneficial in treatment of ischemic ARF.

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*Reprint requests to Dr. Max Hropot, Aventis Pharma Deutschland GmbH, Building H821, 65926 Frankfurt am Main, Germany.*
*E-mail: max.hropo@aventis.com*

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